Vascular Permeability in Experimental Diabetes Is Associated With Reduced Endothelial Occludin Content

Vascular Endothelial Growth Factor Decreases Occludin in Retinal Endothelial Cells

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Blood-retinal barrier (BRB) breakdown is a hallmark of diabetic retinopathy, but the molecular changes that cause this pathology are unclear. Occludin is a transmembrane component of interendothelial tight junctions that may regulate permeability at the BRB. In this study, we examined the effects of vascular endothelial growth factor (VEGF) and diabetes on vascular occludin content and barrier function. Sprague-Dawley rats were made diabetic by intravenous streptozotocin injection, and age-matched animals served as controls. After 3 months, BRB permeability was quantified by intravenous injection of fluorescein isothiocyanate–bovine serum albumin (FITC-BSA), M_r 66 kDa, and 10-kDa rhodamine-dextran (R-D), followed by digital image analysis of retinal sections. Retinal fluorescence intensity for FITC-BSA increased 62% ($P \leq 0.05$), but R-D fluorescence did not change significantly. Occludin localization at interendothelial junctions was confirmed by immunofluorescence, and relative protein content was determined by immunoblotting of retinal homogenates. Retinal occludin content decreased ~35% ($P \leq 0.03$) in the diabetic versus the control animals, whereas the glucose transporter GLUT1 content was unchanged in rat retinas. Additionally, treatment of bovine retinal endothelial cells in culture with 0.12 nmol/l or 12 nmol/l VEGF for 6 h reduced occludin content 46 and 54%, respectively. These data show that diabetes selectively reduces retinal occludin protein expression and increases BRB permeability. Our findings suggest that the elevated VEGF in the vitreous of patients with diabetic retinopathy increases vascular permeability by downregulating occludin content. Decreased tight junction protein expression may be an important means by which diabetes causes increased vascular permeability and contributes to macular edema. ***Diabetes*** **47:1953–1959, 1998**

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BAEC, bovine aortic endothelial cell; BRB, blood-retinal barrier; BREC, bovine retinal endothelial cell; FITC-BSA, fluorescein isothiocyanate–bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; R-D, rhodamine-dextran; STZ, streptozotocin; TER, transendothelial electrical resistance; VEGF, vascular endothelial growth factor.

Breakdown of the blood-retinal barrier (BRB) characterizes early stages of vascular dysfunction in both human and experimental diabetes (1,2). BRB breakdown contributes to vasogenic macular edema that occurs in ~25% of people with diabetes and is highly correlated with visual impairment in people with diabetic retinopathy (3). The mechanism of BRB breakdown has been the focus of numerous reports that have described defects in the inner BRB, including opening of interendothelial cell tight junctions (4,5), endothelial cell transcytosis (6,7), and vacuolation of the retinal pigment epithelium (8,9). These studies used histological methods to examine extravasation of exogenous tracers or endogenous albumin to characterize the loss of BRB integrity. However, the specific molecular pathogenesis for increased permeability has not been elucidated.

The BRB is composed of tight junctions between retinal vascular endothelial cells (inner BRB) and retinal pigment epithelial cells (outer BRB). The normal inner BRB is characterized by well-organized tight junction structures, as seen by electron microscopy, and a paucity of vesicles (10). Clinical studies with differential fluorometry (11) strongly suggest that the inner BRB rather than the outer BRB is the primary site of the vascular leakage that results in macular edema. Therefore, to understand macular edema in diabetic retinopathy, it is necessary to characterize the changes in this barrier at the molecular level.

Tight junctions are comprised of at least seven proteins (12). Occludin is a 65-kDa protein specific to cells that contain tight junctions, and it is thought to span the plasma membrane, conferring the cell-to-cell interaction of tight junctions (13). Expression of the tight junction protein occludin correlates with increased barrier function. Tight junction proteins are expressed in endothelial cells of the blood-brain barrier and BRB (14), and occludin expression has been shown to be specific for vascular endothelial cells with strong barrier properties (15). Furthermore, the expression of tight junction proteins varies in response to stimuli that modulate tissue vascular permeability. We have shown recently that astrocytes

induce barrier properties and increase ZO-1 protein content in retinal endothelial cells (16). In contrast, histamine reduces ZO-1 expression (17). Together, these studies show that, in contrast to the static image of tight junctions suggested by morphological examinations, the molecular composition of tight junctions is highly regulated and changes rapidly in response to factors that affect permeability.

Recent reports have described an increase in permeability factors in the retinas of animal models of diabetes as well as in patients with diabetes. Vascular endothelial growth factor (VEGF) is increased in the retinas of animals with experimental diabetes at 6 months (18) and in patients with nonproliferative diabetic retinopathy (19,20) as well as in the vitreous of patients with proliferative diabetic retinopathy (21). The effect of intravitreal VEGF on vascular permeability in normal rats was blocked with an inhibitor of protein kinase C β (LY 333531), and the same drug also reduces BRB permeability in short-term diabetic rats (22). In addition to VEGF, histamine content and histidine decarboxylase activity are increased in the retinas of rats with experimental diabetes commensurate with increased permeability (23,24). Thus, at least two potent permeabilizing agents, VEGF and histamine, are increased in the retina in diabetes.

We conducted this study to test the hypothesis that experimental diabetes reduces occludin expression coordinately with increased BRB permeability and vasoactive agent content in the retina. Three months of diabetes caused an increase in retinal permeability to albumin and a concomitant decrease in occludin protein content. Furthermore, treatment of primary cultures of retinal endothelial cells with VEGF for 6 h caused a quantitatively similar decrease in occludin content. These findings suggest that elevated VEGF in the retinas of diabetic rats may promote BRB breakdown by reducing vascular endothelial occludin content.

RESEARCH DESIGN AND METHODS

Diabetic animals. Two separate experiments were performed. In experiment one, 14 male Sprague-Dawley rats were used. Of these, six rats served as nondiabetic controls, and eight were made diabetic by a single intravenous injection of streptozotocin (STZ) (65 mg/kg) in 1 mmol/l sodium citrate buffer, pH 4.5. In experiment two, six control rats and six diabetic animals were used. Animals were treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals. They were maintained on a 12-h alternating light/dark cycle and received food and water ad libitum. Animals were declared diabetic when their plasma glucose exceeded 13.7 mmol/l 3 days after STZ injection. Their plasma, glucose, and weight were measured weekly. Diabetic rats received 2–3 U NPH insulin (Lilly, Indianapolis, IN) twice weekly to prevent ketosis. Glycosylated hemoglobin determinations were made at the termination of the experiments using Glycotest II Analytical Columns (Pierce, Rockford, IL) per manufacturer's instructions.

Endothelial cell culture. Primary bovine retinal endothelial cell (BREC) culture was carried out as described previously (17) on 60-mm plates with 2 ml of media. The day before the experiment, cells were stepped down from 20 to 10% fetal calf serum. Recombinant human 165 amino acid VEGF (R&D Systems, Minneapolis, MN) was added to the cell culture by removing 1 ml of media from the plates, combining the media, and adding the appropriate amount of VEGF, then returning the media to the plates. For the experiments described herein, either 500 or 5 ng/ml of VEGF was used, yielding 12 or 0.12 nmol/l VEGF, respectively. Cells were returned to the cell culture incubator for the indicated time, washed with ice-cold phosphate-buffered saline (PBS) containing phenylmethylsulfonyl fluoride (PMSF) (200 μmol/l), and harvested in 6 mol/l urea lysis buffer, as described in the immunoblotting section below.

Measurement of BRB permeability. BRB permeability was measured in experiment one using a modification of the method described by Enea et al. (2). Under ketamine/xylazine anesthesia (80/0.8 mg/kg i.p.), rats received simultaneous tail vein injection of 100 mg/kg each of fluorescein isothiocyanate–bovine serum albumin (FITC-BSA) and 10-kDa rhodamine-dextran (R-D) (Sigma, St. Louis, MO). After 20 min, the animals were killed by decapitation, and the eyes were enucleated. One eye was immediately placed in 10% phosphate-buffered formalin for 48–72 h followed by paraffin wax embedding, and the retina of the second eye was dissected on ice and frozen in liquid nitrogen for biochemical analysis. The time from death to fixation or freezing of the tissues was generally <5 min. At the time of death, blood was collected into EDTA-containing tubes that were centrifuged at 2,000*g* for 10 min. Plasma was assayed for fluorescence with an SPEX fluorescence spectrophotometer (Edison, NJ) based on standard curves of FITC-BSA in normal rat plasma with excitation at 433 ± 2 nm and emission at 455 ± 2 nm, and of R-D with excitation at 578 ± 2 nm and emission at 586 ± 2 nm. FITC and rhodamine are covalently bound to BSA and dextran, respectively, and do not dissociate after intravenous injection or undergo further metabolism, as does sodium fluorescein (2).

The paraffin-embedded eyes were sectioned on a microtome making two 6-μm thick sections every 60 μm. The sections were dewaxed and viewed with an Olympus OM-2 fluorescence microscope fitted with a Sony CLD video camera. Fluorescence intensities of digital images of nonvascular segments of retina were measured using Optimas software on an IBM PC–based image-processing system. Fluorescence intensities of 12 nonvascular retinal points were collected from each section of both control and diabetic rats. The average retinal fluorescent intensity was then normalized to a noninjected control retina analyzed in the same manner and to plasma fluorescence intensity of each animal. Through serial sectioning of each eye, this technique enabled quantification of increased vascular permeability in the retina.

Immunohistochemistry. To determine the cellular expression of occludin, indirect immunohistochemical staining was performed with a rabbit anti-human occludin antibody. Ten-micron cryostat sections were fixed with 2% paraformaldehyde and blocked for 1 h in 10% goat serum in PBS with 0.1% triton. Sections were incubated with rabbit anti-human occludin antibody (Zymed, South San Francisco, CA; 1:2,000) for 16 h at 48°C, washed with PBS 0.1% triton three times for 20 min, and incubated with donkey anti-rabbit secondary antibody conjugated to CY3 (Jackson, West Grove, PA; 1:800) for 1 h at room temperature. A negative control was performed with omission of the primary antibody. In addition, an internal control was used to colocalize occludin immunoreactivity. Barber and Lieth (25) have shown previously that agrin expression, a 225-kDa extracellular matrix protein, is associated with the BRB and the blood-brain barrier. A monoclonal anti-agrin antibody (MAb 33/35; Stressgen, Victoria, BC; 1:2,000) was incubated simultaneously with the anti-occludin antibody, followed by a biotinylated horse anti-mouse secondary antibody (Pierce, Rockford, IL; 1:200) with streptavidin-FITC (Jackson; 1:500). Slides were washed with PBS-triton, and coverslip was mounted with Aquamount (Polysciences, Warrington, PA).

Immunoblotting. Occludin content was quantified by SDS-PAGE and immunoblotting of retinal lysates. Two separate experiments were performed using two different lysis buffers. In experiment one, the retinas were homogenized in Tris-based buffer as described previously (17). Samples were spun for 1 min in the microfuge, an aliquot was used for a protein assay, and another aliquot was combined with Laemmli sample buffer for immunoblotting. In experiment two, retinas were homogenized in urea lysis buffer (6 mol/l urea, 0.1% Triton X-100, 10 mmol/l Tris pH 8.0, 1 mmol/l dithiothreitol, 5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 150 mmol/l NaCl, and 0.2 mmol/l PMSF). Samples were rocked at 48°C for 15 min to solubilize proteins, and insoluble material was pelleted by 10 min centrifugation in microfuge. Protein concentrations were determined by Bio-Rad (Richmond, CA) protein assay, and equal total protein content was loaded onto 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (MSI, Westborough, MA), and immunoblotting for occludin was carried out using anti-human occludin rabbit polyclonal antibody (Zymed, 1:2,000) and detected with ^{125}I-labeled protein A or ^{125}I-labeled goat anti-rabbit antibody (Du Pont-NEN, Wilmington, DE). Alternatively, the blots were detected using an alkaline phosphatase–linked anti-rabbit antibody (1:3,000) and Amersham's (Buckinghamshire, U.K.) enhanced chemifluorescence detection system in conjunction with a 595 fluorimager and Imagequant analysis software (Molecular Dynamics, Sunnyvale, CA). As an internal control, GLUT1 was quantified with a polyclonal anti-human antibody (Santa Cruz, Santa Cruz, CA) at 1:500 dilution. GLUT1 was selected as a control because it is also a membrane-associated protein, and its expression does not change in diabetic rats (26) or in response to histamine in BRECs (17). Membranes were then exposed to radiographic film, and occludin content was quantified by scanning the autoradiograms (Microtek scanner connected to Macintosh with Adobe Photoshop 3.0) and image analysis (NIH Image, V. 1.61).

Statistical methods. Change in animal weight or percent glycosylated hemoglobin was compared with unpaired Student's t test using a two-tailed P value or, if the SDs were significantly different, by Mann-Whitney test. The mean intensities of fluorescence were compared by the Mann-Whitney test after standardizing to a nonfluorescent control. Occludin content was compared using unpaired Student's t test with a two-tailed P value. In all cases, α was set at 0.05.

TABLE 1
Weight change and HbA_{1c} of control and diabetic rats

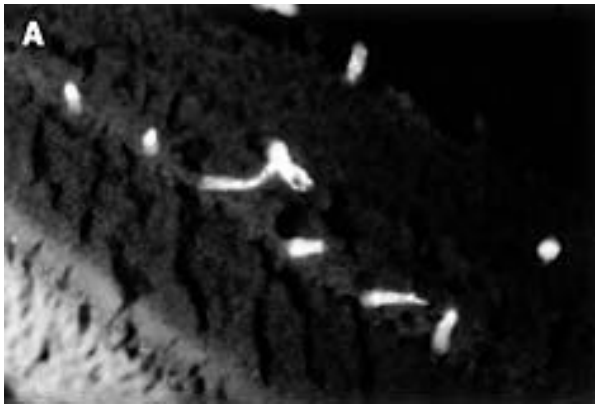
	Animals	*n*	% Weight change	% HbA_{1c}
Experiment 1	Control	6	43.8 ± 2.73	3.6 ± 0.20
	Diabetic	8	–22.1 ± 5.89*	13.76 ± 0.82*
Experiment 2	Control	6	57.57 ± 4.28	3.42 ± 0.27
	Diabetic	6	6.68 ± 4.82†	13.05 ± 0.64†

Data are means + SE. Animals were made diabetic by a single STZ injection (65 mg/kg) in 1 mmol/l sodium citrate buffer, pH 4.5. Diabetic rats received 2–3 U NPH insulin twice weekly to prevent ketosis. Starting whole-body weights were 526 ± 17 and 490 ± 34 g for control and diabetic groups, respectively, in experiment 1 and 432 ± 33.9 and 511 ± 11 g for control and diabetic groups, respectively, in experiment 2. HbA_{1c} determinations were made at the termination of the experiments. *Significantly different from control (Mann-Whitney, $P < 0.001$); †significantly different from control (Student's t test, $P < 0.0001$).

RESULTS

Male Sprague-Dawley rats were made diabetic by intravenous tail-vein injection of STZ. As expected, after 3 months of experimental diabetes, the glycosylated hemoglobin content increased significantly to nearly fourfold of the control condition. Furthermore, the body weights of the diabetic rats decreased 22% over the 3 months of diabetes, while body weights of the control animals increased 44% (Table 1). These values are similar to those reported previously (2).

BRB permeability. Increased BRB permeability is an early event in diabetes (1) and continues with progression of retinopathy (27). BRB permeability was assessed with FITC-BSA because albumin has previously been demonstrated to be permeant in early diabetes (2), moves through interendothelial cell junctions (28,29), and has a molecular mass (66 kDa) that is similar to that of macromolecules, such as lipoproteins, that accumulate in the retina in diabetic retinopathy. This technique has the advantage over immunodetection methods in that it does not rely on antibody-binding affinities, is directly quantifiable, and background fluorescence is negligible. Figure 1 shows representative fluorescence micrographs of FITC-BSA in control and diabetic rat eyes. The fluorescence intensity is limited to the vasculature in the control retinas (*A*) and diffusely increased throughout the retinal parenchyma in the diabetic retina (*B*). These findings confirm that BRB permeability to albumin is increased early in experimental diabetes. Figure 1*C* shows the change in FITC-BSA fluorescence intensity in control and diabetic retinas after nor-



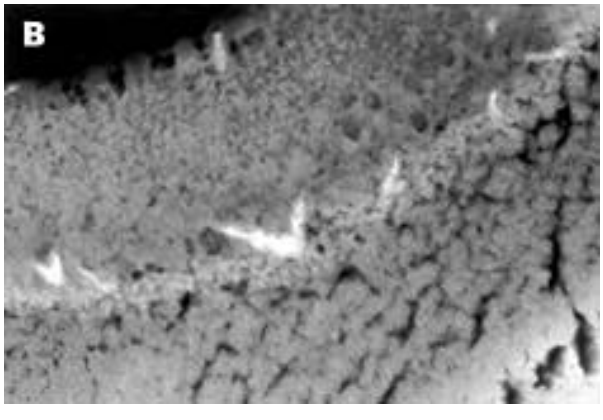
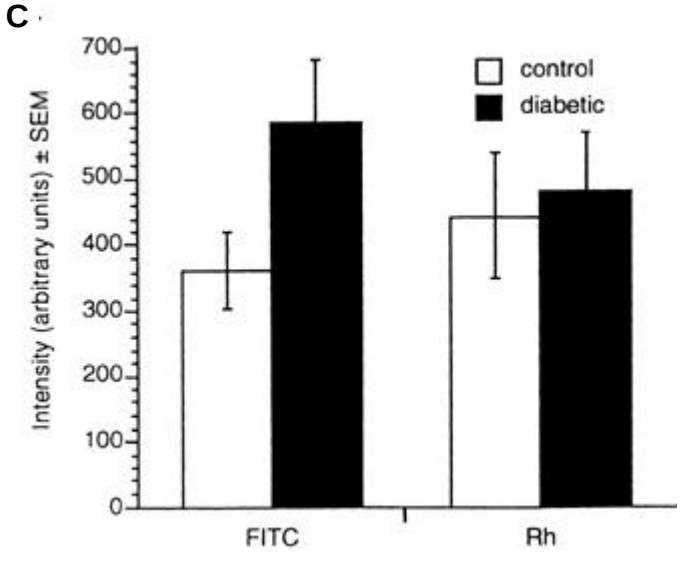


FIG. 1. FITC fluorescence in diabetic and control rat retina. In sections of retina from control rats (*A*), FITC fluorescence was limited to the lumens of blood vessels, whereas in sections from diabetic rats (*B*), FITC fluorescence was distributed evenly throughout the retinal parenchyma. Permeability measurements of fluorescence in serial sections of rat retina are presented graphically (*C*). STZ-induced diabetic rats or control rats were injected intravenously with a mixture of FITC-BSA and 10 kDa R-D (100 mg/kg each). The degree of retinal fluorescence was measured by image analysis in serial sections on paraffin-embedded eyes. Diabetic rats had 62% more FITC fluorescence compared with controls ($P < 0.05$), but there was no significant increase in the amount of rhodamine fluorescence. Original magnification ×200.



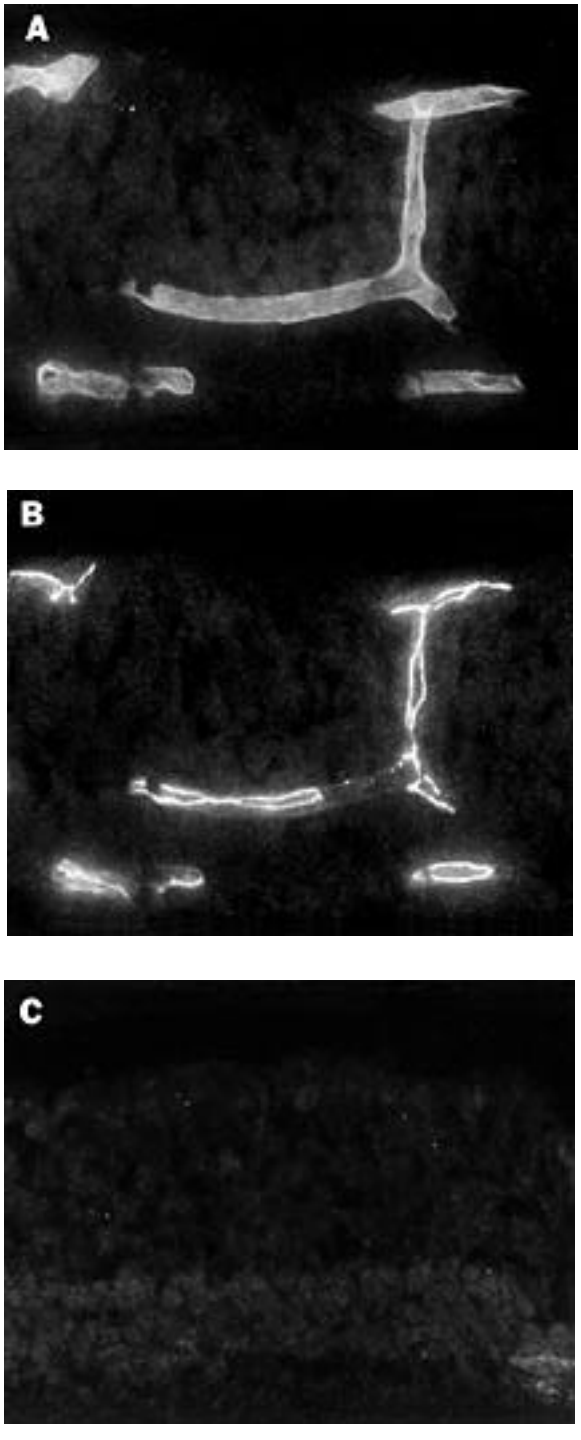


FIG. 2. Occludin immunoreactivity in rat retina. Retinal vessels identified by positive agrin immunoreactivity (*A*) were also immunoreactive for occludin (*B*). The occludin immunoreactivity had a distribution consistent with tight junctions. A negative control in which primary antibody was omitted had essentially no immunoreactivity (*C*). Original magnification ×200.

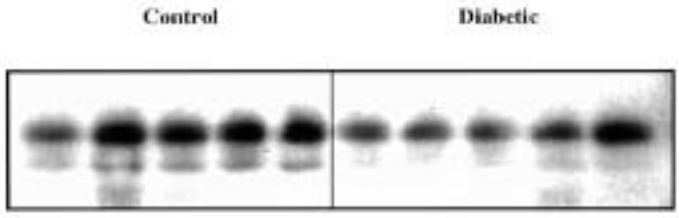


FIG. 3. Experimental diabetes reduces occludin content. Homogenates of rat retinas in Tris-based buffer were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted for occludin. Occludin content is reduced in the diabetic group by 34%, whereas GLUT1 content did not change (not shown). Similar results were found with retinas homogenized in urea-based buffer.

malizing to plasma fluorescence. Diabetes increased retinal FITC-BSA fluorescence 62% ($P < 0.05$). The observed increase was larger than, but comparable to, the 25% increase in ^{125}I-labeled albumin permeability observed by Williamson et al. (30) after 3 weeks of experimentally induced diabetes. We also measured the permeability to a smaller molecule, 10-kDa R-D, in the same retinas. In contrast to FITC-BSA, the lower molecular weight but more hydrophilic 10-kDa R-D was not more permeant in the diabetic animals. This data suggests that BRB permeability depends not only on molecular mass but also on additional physical properties of the molecule.

Occludin expression in retinas. To examine the distribution of retinal occludin expression, indirect immunohistochemistry was performed with colocalization for agrin. As shown in Fig. 2, occludin expression is confined to the inner and outer layers of retinal vessels and highlights the interendothelial cell junctions. The vascular expression of occludin was confirmed by colocalization with agrin immunoreactivity. A negative control with no primary antibody revealed no immunoreactivity on retinal vessels. These results are consistent with the model of the inner BRB maintained by the retinal vascular endothelium. As reported previously (25), agrin expression is also confined to the retinal microcirculation, and its distribution is similar to that of laminin.

The expression of the tight junction protein, occludin, most closely correlates with the formation of strong barrier properties. To determine whether experimental diabetes changes the expression of occludin in the retina, quantitative immunoblotting was performed on retinal lysates obtained from either a Tris/SDS-based extraction buffer or a urea-based extraction buffer. Occludin content was reduced after 3 months of experimental diabetes in both experiments by 34 and 32%, respectively (Fig. 3 and Table 2). GLUT1 content did not change (not shown). The fact that two different extraction techniques yielded similar results suggests that the reduction in occludin immunoreactivity represents decreased cellular content rather than a decrease in a specific subpopulation of occludin. The results reveal that experimentally induced diabetes decreases occludin expression at a time when BRB permeability is increased.

Occludin expression in BRECs. Because VEGF is increased in the eyes of animals with experimental diabetes, we wished to determine whether the decrease in occludin content observed in the diabetic rats could result from a chronic exposure to this permeabilizing hormone. Primary BRECs were incubated in the presence of 0.12 or 12 nmol/l VEGF. Treatment of the cells with VEGF caused a 46 and 54% decrease in occludin content after 6 h ($P \leq 0.0001$ and $P \leq 0.009$), as determined by Western immunoblotting (Fig. 4). Again, GLUT1 content was not affected by VEGF treatment (data not shown). A radioimmunoassay of VEGF in the media

TABLE 2
Occludin content in control and diabetic rat retinas

	Animals	*n*	Relative occludin expression
Experiment 1 (Tris/SDS)	Control	5	1 ± 0.11
	Diabetic	5	0.66 ± 0.06*
Experiment 2 (Urea)	Control	6	1 ± 0.08
	Diabetic	6	0.68 ± 0.09*

Data are means ± SE for the amount of occludin expressed relative to the control animals. Relative occludin expression was determined by immunoblotting total cell protein separated on 7.5% denaturing gels. *Significantly different from control (Student's *t* test, $P \leq 0.03$).

revealed no significant change in VEGF content over the 6-h time course of the experiment. Thus, chronic exposure of endothelial cells to VEGF specifically reduces occludin content as determined by immunoreactivity.

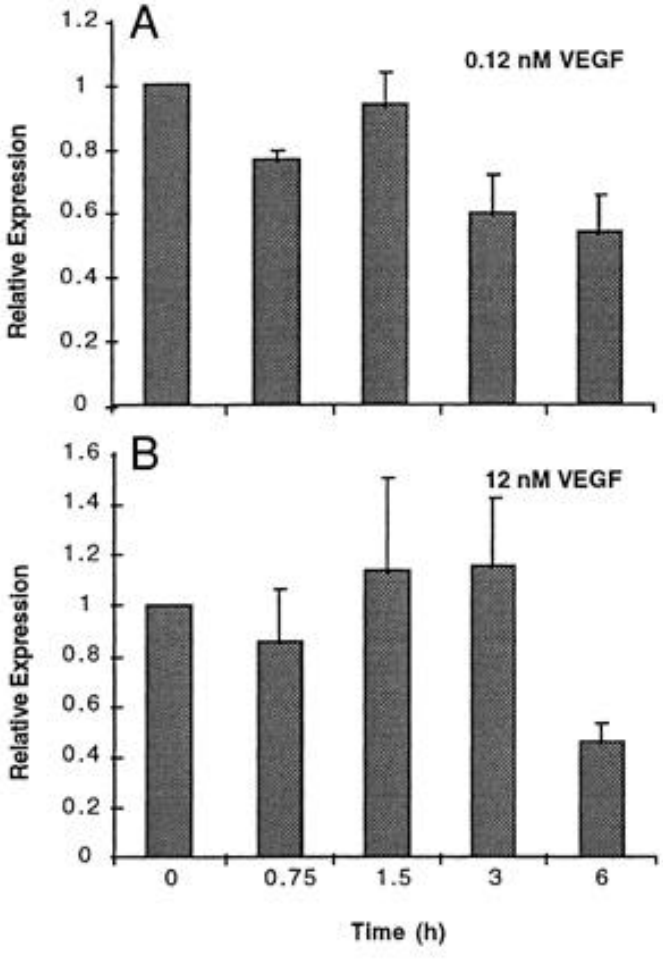


FIG. 4. VEGF reduces occludin content in BRECs. BREC cultures were treated with either 0.12 (*A*) or 12 (*B*) nmol/l VEGF, and cells were harvested in a urea-based buffer at the indicated time points. Proteins were separated by gel electrophoresis on a 7.5% gel and transferred to nitrocellulose. Occludin was detected by immunoblotting, and the relative content to the control (0 h VEGF) was determined. The results presented are combined from two separate experiments yielding an *n* of 4 or 5 for each time point. There was a significant decrease in occludin after 0.12 nmol/l VEGF for 3 h ($P < 0.002$) and 6 h ($P < 0.0001$) and after 12 nmol/l VEGF for 6 h ($P < 0.009$, unpaired Student's *t* test). Both experiments yielded a similar change in occludin content.

DISCUSSION

BRB breakdown is a hallmark of early diabetic retinopathy (1,2). The major findings of this report are *1*) that the content of the membrane-spanning tight junction protein, occludin, decreases in early experimental diabetes at a time when BRB permeability is increased and *2*) that VEGF specifically decreases occludin content in BRECs. Together, these data strongly suggest that the increase in the BRB permeability to albumin could be caused by the observed decrease in immunoreactive occludin.

Several recent reports reveal the role of occludin in establishing a relatively impermeable tissue barrier. Hirase et al. (15) used immunohistochemistry to show that occludin expression is highest in vessels with tight barriers, such as vasculature in the brain, and undetectable in much more permeable endothelial cells, such as bovine aortic endothelial cells (BAECs). By Western blot analysis, we have also observed that occludin content is very low, but detectable, in BAEC cultures (L. DeMaio, unpublished observations), whereas its expression is readily detectable in BRECs. Additionally, overexpression of occludin by transfection increases transendothelial electrical resistance (TER) in the Madin Darby canine kidney cell line (31,32) and cell adhesion in rat 1 fibroblasts (33). Microinjection of COOH-terminal truncated occludin into oocytes causes a dominant negative effect on macromolecular tissue barrier formation (34). Finally, extended incubation of A6 Xenopus kidney epithelial cells with a synthetic peptide corresponding to the extracellular domain of occludin decreases TER and increases paracellular flux of molecules up to 3,000 Da concurrent with a reduction in occludin content (35). Taken together, these findings suggest that alterations in occludin protein expression may be a mechanism by which tissue permeability is regulated. However, the mechanism of barrier regulation is probably more complex than a direct relation of occludin content, since overexpression of occludin, while increasing TER, could also cause an increase in the permeability of small molecule flux (31,32), and an occludin knockout mouse is viable (36).

BRB permeability in diabetes probably increases because of vasoactive factors, such as VEGF/vascular permeability factor (18–20,22) and histamine (2,37). We have shown recently that histamine reduces expression of the tight junction/adherens junction protein, ZO-1, in cultures of retinal endothelial cells (17). Furthermore, the expression of ZO-1 can be upregulated by astrocytes in BRECs coincident with an increase in TER (16). To our knowledge, the results presented herein are the first example of a pathophysiological

change in occludin content coincident with a change in barrier permeability function. The reduction in occludin content observed in rats with experimental diabetes coincident with a decrease in BRB permeability suggests that the decrease in occludin protein content directly results in increased BRB permeability. In humans, this could contribute to the macular edema observed in diabetic retinopathy. The reduction in occludin content caused by VEGF on BRECs suggests that this may be a direct effect of the increased VEGF content in the retinas of diabetic patients. The focus of this study was to examine chronic effects of diabetes and VEGF. Clearly, VEGF also increases BRB permeability more rapidly than the 6-h change in occludin content described herein. However, the observed long-term changes in occludin content do not preclude any rapid modifications in tight junctions that could mediate immediate changes in paracellular permeability. The mechanism by which VEGF reduces occludin expression is currently under investigation in our laboratories.

The direct observation in this report is a decrease in occludin immunoreactivity by Western blot analysis. This was observed by using both Tris/SDS- and urea-based extraction techniques, and, therefore, it is unlikely that it represents a change in occludin localization. Although the samples were separated on denaturing SDS-polyacrylamide gels, it is still formally possible that the decrease in immunoreactivity represents a change in protein conformation not denatured by the gel technique.

The observation that albumin permeability was increased but that 10-kDa dextran permeability was not raises several possible interpretations. It is unlikely that the 10-kDa R-D extravasated from the retina, because the eyes were promptly fixed in formalin. Moreover, the ability to observe sodium fluorescein permeability in response to VEGF injection in rat eyes (22) also makes this possibility unlikely. Therefore, we suspect that the tight junctions may selectively regulate molecular permeability dependent on molecule size as well as other factors, such as lypophilicity. It is well established that strongly lipophilic molecules, such as theophylline, are able to pass across intact blood-tissue barriers. The reduction in occludin content may directly cause or reflect a change in the ability of tight junctions to maintain a highly selective permeability barrier. Thus, moderately lipophilic molecules, such as albumin, which were previously excluded, can now pass across the barrier, whereas large strongly hydrophilic molecules, such as 10-kDa dextran, remain impermeant. Tumor necrosis factor decreases TER in epithelial cells, reflecting a change in water permeability; however, although permeability increases for smaller lipophilic molecules, 10-kDa dextran remained impermeant (38). Additionally, a decrease in occludin content of ~80% in A6 epithelial cells, by extended incubation of a peptide to an extracellular domain of occludin, increased permeability to dextran 3,000 but only marginally increased permeability to dextran 40,000 and markedly decreased TER (35). Furthermore, selective albumin escape occurs in the cerebral cortex of rats with 2 weeks of diabetes (39). From a clinical standpoint, patients with diabetic retinopathy can have retinal lipid exudates without retinal thickening or serous fluid accumulation (T.W.G., unpublished observations), and urine albumin excretion precedes leakage of low molecular weight dextrans in the earliest stages of type 1 diabetes (40). Thus, albumin permeability may be an early indication of vascular barrier breakdown in diabetes.

It is also possible that the increased albumin permeability in diabetes is due to increased transcytosis. Several previous electron microscopic studies of tracers in the retinal vascular endothelium of animal models with diabetes have been reported in which the route of albumin leakage into the retina was investigated. While two reports identified definite open interendothelial junctions with horseradish peroxidase (4,5), Vinores et al. (6) did not detect albumin in open tight junctions in rat or human diabetic retinopathy. Immunohistochemistry for albumin has the advantage of avoiding exogenous tracers, but morphological observations alone cannot exclude the existence of functional tight junction defects. Opening of even a small number of tight junctions in a vascular bed may significantly increase permeability to molecules. For example, albumin may leak at foci of relatively large gaps in endothelial cell tight junctions or at sites of reduced tight junction formation, which may occur with a decrease in occludin content (41). We have shown recently that VEGF increases hydraulic conductivity, a measure of paracellular permeability, in BREC monolayers (42). Luna et al. (43), using transmission electron microscopy, found that intravitreal VEGF in normal rabbit eyes significantly opened endothelial cell tight junctions, with the maximal observed effect at 6 h and a spontaneous closure by 24 h. Together with our results, these observations suggest that paracellular flux contributes to increased vascular permeability in diabetes. However, it is important to point out that changes in paracellular flux and transcytosis are not mutually exclusive. In fact, tight junctions define the vascular apical versus basolateral environment and thus may contribute to directing transcellular flux. Moreover, the current study does not prove that changes in occludin content per se account for increased permeability. Other tight junction protein molecules, notably 7H6, have been correlated with endothelial cell permeability (44). Nevertheless, several studies have suggested that occludin changes inversely with various measures of tight junction integrity, and the magnitude of increased BRB permeability in this report is consistent with the observed reduction in occludin content. These findings, *1*) that occludin content is decreased at an early point of experimental diabetes in the rat retinal vasculature and *2*) that VEGF decreased retinal endothelial cell occludin content, make it likely that VEGF-mediated changes in expression of occludin in retinal vascular endothelial cells account for at least some of the increased permeability observed in experimental and human diabetic retinopathy.

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Author Queries (please see Q in margin and underlined text)

Q1: Please define ARVO.

Q2: Goat anti-rabbit “antibody” OK?

Q3: Please provide names of researchers of unpublished observations, initials and full last name of those who are not authors of this study.

Q4: Define VPF.